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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation)

INTRODUCTION

1. This Test Guideline (TG) describes a method to determine the effects of a substance on micro-organisms from activated sludge (largely bacteria) by measuring their respiration rate (carbon and/or ammonium oxidation) under defined conditions in the presence of different concentrations of the test substance. The method is based on the ETAD (Ecological and Toxicological Association of the Dyestuffs Manufacturing industry) test (1) (2), on the existing OECD Test Guideline 209 (3) and on the revised ISO Standard 8192 (4). The purpose of the test is to provide a rapid screening method to assess the effects of substances on the microorganisms of the activated sludge of the biological (aerobic) stage of waste-water treatment plants. The results of the test may also serve as an indicator of suitable non-inhibitory concentrations of test substances to be used in biodegradability tests (for example OECD TG 301 series, TG 310, TG 302 series and TG 303). In this case, the test can be performed as a screening test, similar to a range-finding or limit test (see Paragraph 39), considering the overall respiration only. However, this information should be taken with care for ready biodegradability tests (OECD TG 301 series and TG 310) for which the inoculum concentration is significantly lower than the one used in this Test Guideline. Indeed, an absence of inhibition in this respiration test does not automatically result in non-inhibitory conditions in the ready biodegradability test of the TG 301 series or TG 310.

2. Overall, the respiration inhibition test seems to have been applied successfully since it was first published, but on some occasions spurious results were reported, e.g. (2) (4) (5). Concentration related respiration curves are sometimes bi-phasic, dose-response plots have been distorted and EC_{50} values have been unexpectedly low (5). Investigations showed that such results are obtained when the activated sludge used in the test nitrifies significantly and the test substance has a greater effect on the oxidation of ammonium than on general heterotrophic oxidation. Therefore, these spurious results may be overcome by performing additional testing using a specific inhibitor of nitrification. By measuring the oxygen uptake rates in the presence and absence of such an inhibitor, e.g. N-allylthiourea (ATU), the separate total, heterotrophic and nitrification oxygen uptake rates can be calculated (4) (7) (8). Thus, the inhibitory effects of a test substance on the two processes may be determined and the EC_{50} values for both organic carbon oxidation (heterotrophic) and ammonium oxidation (nitrification) may be calculated in the usual way. It should be noted that in some rare cases, the inhibitory effect of N-allylthiourea may be partially or completely nullified as a result of complexation with test substances or medium supplements, e.g. Cu^{++} ions (6). Cu^{++} ions are essential for *Nitrosomonas*, but are toxic in higher concentration.

3. The need for nitrification in the aerobic treatment of wastewaters, as a necessary step in the process of removing nitrogen compounds from wastewaters by denitrification to gaseous products, has become urgent particularly in European countries; the EU has now set lower limits for the concentration of nitrogen in treated effluents discharged to receiving waters.

4. For most purposes, the method to assess the effect on organic carbon oxidation processes alone is adequate. However, in some cases an examination of the effect on nitrification alone, or on both

nitrification and organic carbon oxidation separately, are needed for the interpretation of the results and understanding the effects.

PRINCIPLE OF THE TEST

5. The respiration rates of samples of activated sludge fed with synthetic sewage are measured in an enclosed cell containing an oxygen electrode after a contact time of 3 hours. Under consideration of the realistic exposure scenario, longer contact times could be appropriate. If the test substance is rapidly degraded e.g. abiotically via hydrolysis, or is volatile and the concentration cannot be adequately maintained, additionally a shorter exposure period e.g. 30 minutes can be used. The sensitivity of each batch of activated sludge should be checked with a suitable reference substance on the day of exposure. The test is typically used to determine the EC_x (e.g. EC_{50}) of the test substance and/or the no-observed effect concentration (NOEC).

6. The inhibition of oxygen uptake by micro-organisms oxidising organic carbon may be separately expressed from that by micro-organisms oxidising ammonium by measurement of the rates of uptake of oxygen in the absence and presence of N-allylthiourea, a specific inhibitor of the oxidation of ammonium to nitrite by the first-stage nitrifying bacteria. In this case the percentage inhibition of the rate of oxygen uptake is calculated by comparison of the rate of oxygen uptake in the presence of a test substance with the mean oxygen uptake rate of the corresponding controls containing no test substance, both in the presence and absence of the specific inhibitor, N-allylthiourea.

7. Any oxygen uptake arising from abiotic processes may be detected by determining the rate in mixtures of test substance, synthetic sewage medium and water, omitting activated sludge.

INFORMATION OF THE TEST SUBSTANCE

8. The identification (preferably CAS number), name (IUPAC), purity, water solubility, vapour pressure, volatility and adsorption characteristics of the test substance should be known to enable correct interpretation of results to be made. Normally, volatile substances cannot be tested adequately unless special precautions are taken (see paragraph 21).

APPLICABILITY OF THE METHOD

9. The test method may be applied to water-soluble, poorly soluble and volatile substances. However, it may not always be possible to obtain EC_{50} values with chemicals of limited solubility and valid results with volatile chemicals may only be obtained providing that the bulk (say > 80%) of the test substance remains in the reaction mixture at the end of the exposure period(s). Additional analytical support data should be submitted to refine the EC_x concentration when there is any uncertainty regarding the stability of the test substance or its volatility.

REFERENCE SUBSTANCES

10. Reference substances should be tested periodically in order to assure that the test method and test conditions are reliable, and to check the sensitivity of each batch of activated sludge used as microbial inoculum on the day of exposure. The chemical 3,5-dichlorophenol (3,5-DCP) is recommended as the reference inhibitory substance, since it is a known inhibitor of respiration and is used in many types of test for inhibition/toxicity (4). Also copper (II) sulphate pentahydrate can be used as a reference substance for the inhibition of total respiration (9). N-methylaniline can be used as a specific reference inhibitor of nitrification (4).

VALIDITY CRITERIA AND REPRODUCIBILITY

11. The blank controls (without the test substance or reference substance) oxygen uptake rate should not be less than 20 mg oxygen per one gram of activated sludge (dry weight of suspended solids) in an hour. If the rate is lower, the test should be repeated with washed activated sludge or with the sludge from another source. The coefficient of variation of oxygen uptake rate in control replicates should not be more than 30% at the end of definitive test.

12. In a 2004 international ring test organized by ISO (4) using activated sludge derived from domestic sewage, the EC_{50} of 3,5-DCP was found to lie in the range 2 mg/L to 25 mg/L for total respiration, 5 mg/L to 40 mg/L for heterotrophic respiration and 0.1 mg/L to 10 mg/L for nitrification respiration. If the EC_{50} of 3,5-DCP does not lie in the expected range, the test should be repeated with activated sludge from another source. The EC_{50} of copper (II) sulphate pentahydrate should lie in the range of 53-155 mg/L for the total respiration (9).

DESCRIPTION OF THE TEST METHOD

Test vessels and apparatus

13. Usual laboratory equipment and the following should be used:

(a) Test vessels – for example, 1000 mL beakers to contain 500 mL of reaction mixture (see 5 in Fig.1);

(b) Cell and attachments for measuring concentration of dissolved oxygen; a suitable oxygen electrode; an enclosed cell to contain the sample with no headspace and a recorder (e.g. 7, 8, 9 in Fig.1 of Annex 2); alternatively, a BOD bottle may be used with a suitable sleeve adaptor for sealing the oxygen electrode against the neck of the bottle (see Fig. 2 of Annex 3). To avoid loss of displaced liquid on insertion of the oxygen electrode, it is advisable first to insert a funnel or glass tube through the sleeve, or to use vessels with flared-out rims. In both cases a magnetic stirrer or alternative stirrer method, e.g. self-stirring probe, should be used;

(c) Magnetic stirrers and followers, covered with inert material, for use in measurement chamber and/or in the test vessels;

(d) Aeration device: if necessary, compressed air should be passed through an appropriate filter to remove dust and oil and through wash bottles containing water to humidify the air. The contents of vessels should be aerated with Pasteur pipettes, or other aeration devices, which do not adsorb chemicals. An orbital shaker operated at orbiting speeds between 150 and 250 rpm with flasks of, for example, 2000 mL capacity, can be used to satisfy the oxygen demand for the sludge and overcome difficulties with substances that produce excessive foam, are volatile and therefore lost, or are difficult to disperse when aerated by air sparging. The test system is typically a number of beakers aerated continuously and sequentially established (e.g. at *ca*. 10 – 15 minute intervals), then analysed in a sequential manner. Validated instrumentation that allows the simultaneous aeration and measurement of the oxygen consumption rate in the mixtures may also be used;

(e) pH-meter;

(f) Centrifuge, general bench-top centrifuge for sludge capable of 10,000 m/s^2.

Reagents

14. Analytical grade reagents should be used throughout.

Water

15. Distilled or deionised water, containing less than 1mg/L DOC, should be used except where chlorine free tap water is specified.

Synthetic sewage feed

16. The medium should be prepared to contain the following constituents at the stated amounts:

- peptone	16 g
- meat extract (or a comparable vegetable extract)	11 g
- urea	3 g
- sodium chloride (NaCl)	0.7 g
- calcium chloride dihydrate ($CaC1_2$, $2H_2O$)	0.4 g
- magnesium sulphate heptahydrate ($MgSO_4$, $7H_2O$)	0.2 g
- anhydrous potassium monohdydrogen phosphate (K_2HPO_4)	2. 8g
- distilled or deionized water to 1 litre	

17. The pH of this solution should be 7.5 ± 0.5. If the prepared medium is not used immediately, it should be stored in the dark at 0^oC to 4^oC, for no longer than 1 week or under conditions, which do not change its composition. It should be noted that this synthetic sewage is a 100 fold concentrate of that described in the OECD Technical Report "Proposed method for the determination of the biodegradability of surfactants used in synthetic detergents" June 11, 1976, with moreover dipotassium hydrogen phosphate added.

18. Alternatively, components of the medium can be sterilized individually prior to storage, or the peptone and meat extract can be added shortly before carrying out the test. Prior to use, the medium should be thoroughly mixed and the pH adjusted if necessary to pH 7.5 ± 0.5.

Test substance

19. A stock solution should be prepared for readily water soluble test substances up to the maximum water solubility only (precipitations are not acceptable). Poorly water soluble substances, mixtures with components of different water solubility and adsorptive substances should be directly weighed into the test vessels. In these cases, use of stock solutions may be an alternative if dissolved concentrations of the test substances are analytically determined in the test vessels (prior to adding activated sludge). If water accommodated fractions (WAFs) are prepared, an analytical determination of the dissolved concentrations of the test substances in the test vessels is also essential. Using organic solvents, dispersants/emulsifiers to improve solubility should be avoided. Ultrasonication of stock solutions and pre-stirring suspensions, e.g. overnight, is possible when there is adequate information available concerning the stability of the test substance under such conditions.

20. The test substance may adversely affect pH within the test system. The pH of the test substance-treated mixtures should be determined prior to the test set up, in a preliminary trial, to ascertain whether pH adjustment will be necessary prior the main test and again on the day of the main test. Solutions/ suspensions of test substance in water should be neutralised prior to inoculum addition, if necessary. However, since neutralisation may change the chemical properties of the substance, further testing,

depending on the purposes of the study, could be performed to assess the effect of the test substance on the sludge without pH adjustment.

21. The toxic effects of volatile substances, especially in tests in which air is bubbled through the system, can result in variable effect levels occurring owing to losses of the substance during the exposure period. Caution should be exercised with such substances by performing substance specific analysis of control mixtures containing the substance and modifying the aeration regime.

Reference substance

22. If 3,5-dichlorophenol is used as reference substance, a solution of 1.00 g of 3,5-dichlorophenol in 1000 mL of water should be prepared (15). Warm water and/or ultrasonication should be used to accelerate the dissolution and make the solution up to volume when it has cooled to room temperature. However, it should be ensured that reference substance is not structurally changed. The pH of the solution should be checked and adjusted, if necessary, with NaOH or H_2SO_4 to pH 7 – 8.

23. If copper(II)sulphate pentahydrate is used as a reference substance, concentrations of 58 mg/L, 100 mg/L and 180 mg/L (a factor of 1.8) are used. The substance is weighed in directly into the test vessels (29 – 50 – 90 mg for 500 mL total volume). It is then dissolved with 234 mL of autoclaved tap water. Copper(II)sulphate pentahydrate is easily soluble. When the test is started, 16 mL of synthetic sewage and 250 mL of activated sludge are added.

Specific inhibitor of nitrification

24. A 2.32 g/L stock solution of N-allylthiourea (ATU) should be prepared. The addition of 2.5 mL of this stock solution to an incubation mixture of final volume of 500 mL results in a final concentration of 11.6 mg ATU/L (10^{-4}mol/L) which is known to be sufficient (4) to cause 100% inhibition of nitrification in a nitrifying activated sludge containing 1.5g/L suspended solids.

Abiotic control

25. Under some rare conditions, a test substance with strong reducing properties may cause measurable abiotic oxygen consumption. In such cases, abiotic controls are necessary to discriminate between abiotic oxygen uptake by the test substance and microbial respiration. Abiotic controls may be prepared by omitting the inoculum from the test mixtures. Similarly, abiotic controls without inoculum may be included when supporting analytical measurements are performed to determine the achieved concentration during the exposure phase of the test, e.g. when using stock solutions of poorly water soluble substances or mixtures with components with different water solubility. In specific cases it may be necessary to prepare an abiotic control with sterilized inoculum (e.g. by autoclaving or adding sterilizing toxicants). Some substances may produce or consume oxygen only if the surface area is big enough for reaction, even if they normally need a much higher temperature or pressure to do so. In this respect special attention should be given to peroxy substances. A sterilized inoculum provides a big surface area.

Inoculum

26. For general use, activated sludge should be collected from the exit of the aeration tank, or near the exit from the tank, of a well-operated wastewater treatment plant receiving predominantly domestic sewage. Depending on the purpose of the test, other adequate types or sources of activated sludge, e.g. sludge grown in the laboratory, may also be used at suitable suspended solids concentrations of 2 g/L to 4 g/L. However, sludges from different treatment plants are likely to exhibit different characteristics and sensitivities.

27. The sludge may be used as collected but coarse particles should be removed by settling for a short period, e.g. 5 to 15 minutes, and decanting the upper layer of finer solids or sieving (e.g. 1 mm^2 mesh). Alternatively, the sludge may be homogenized in a blender for a *ca.* 15 seconds or longer, but caution is needed regarding the shear forces and the temperature change which might occur for long periods of blending.

28. Washing the sludge is often necessary, e.g. if the endogenous respiration rate is low. The sludge should first be centrifuged for a period to produce a clear supernatant and pellet of sewage solids e.g. 10 minutes at ca.10, 000 m/s^2. The supernatant liquid should be discarded and the sludge re-suspended in chlorine-free tap water, with shaking, and the wash-water should then be removed by re-centrifuging and discarding again. The washing and centrifuging process should be repeated, if necessary. The dry mass of a known volume of the re-suspended sludge should be determined and the sludge concentrated by removing liquor or diluted further in chlorine-free tap water to obtain the required sludge solids concentration of 3 g/L. The activated sludge should be continuously aerated (e.g. 2 L/minute) at the test temperature and, where possible used on day of collection. If this is not possible, the sludge should be fed daily with the synthetic sewage feed (50 mL synthetic sewage feed/L activated sludge) for two additional days. The sludge is then used for the test and the results are accepted as valid, provided that no significant change in its activity, assessed by its endogenous heterotrophic and nitrification respiration rate, has occurred.

29. Difficulties can arise if foaming occurs during the incubation to the extent that the foam and the sludge solids carried on it, are expelled from the aeration vessels. Occasionally, foaming may simply result from the presence of the synthetic sewage, but foaming should be anticipated if the test substance is, or contains, a surfactant. Loss of sludge solids from the test mixtures will result in artificially lowered respiration rates that could mistakenly be interpreted as a result of inhibition. In addition, aeration of surfactant solution concentrates the surfactant in the foam layer; loss of foam from the test system will lower the exposure concentrations. The foaming can be controlled by simple mechanical methods (e.g. occasional manual stirring using a glass rod) or by adding a surfactant-free silicone emulsion antifoam agent and/or use the shake flask aeration method. If the problem is associated with the presence of the synthetic sewage, the sewage composition should be modified by including an antifoam reagent at a rate of e.g. 50 µl/L. If foaming is caused by the test substance, the quantity needed for abatement should be determined at the maximum test concentration, and then all individual aeration vessels should be identically treated (including those, e.g. blank controls and reference vessels where foam is absent). If antifoam agents are used, there should be no interaction with inoculum and/or test substance.

TEST PROCEDURE

30. The inhibition of three different oxygen uptakes may be determined, total, heterotrophic only and that due to nitrification. Normally, the measurement of total oxygen uptake inhibition should be adequate. The effects on heterotrophic oxygen uptake from the oxidation of organic carbon, and due to the oxidation of ammonium are needed when there is a specific requirement for such two separate end-points for a particular substance or (optionally) to explain atypical dose-response curves from inhibition of total oxygen uptake.

Test conditions

31. The test should be performed at a temperature within the range 20±2°C.

Test mixtures

32. Test mixtures (F_T as in Table 1) containing water, synthetic sewage feed and the test substance should be prepared to obtain different nominal concentrations of the test substance (See Table 1 for example of volumes of constituents). The pH should be adjusted to 7.5 ± 0.5, if necessary; mixtures should be diluted with water and the inoculum added to obtain equal final volumes in the vessels and to begin the aeration.

Reference mixtures

33. Mixtures (F_R) should be prepared with the reference compound, e.g. 3,5-dichlorophenol, in place of the test substance in the same way as the test mixtures.

Blank controls

34. Blank controls (F_B) should be prepared at the beginning and end of the exposure period in tests in which the test beakers are set up sequentially at intervals. In tests performed using equipment which allows simultaneous measurements of oxygen consumption to be made, at least two blank controls should be included in each batch of simultaneous analysis. Blank controls contain an equal volume of activated sludge and synthetic medium but not test or reference substance. They should be diluted with water to the same volume as the test and reference mixtures.

Abiotic control

35. If necessary, for example if a test substance is known or suspected to have strong reducing properties, a mixture F_A should be prepared to measure the abiotic oxygen consumption. The mixture should have the same amounts of test substance, synthetic sewage feed and the same volume as the test mixtures, but no activated sludge.

General procedure and measurements

36. Test mixtures, reference mixtures and the blank and abiotic controls are incubated at the test temperature under conditions of forced aeration (0.5 to 1 L/min) to keep the dissolved oxygen concentration above 60 – 70% saturation and to maintain the sludge flocs in suspension. Stirring the cultures is also necessary to maintain sludge flocs in suspension. The incubation is considered to begin with the initial contact of the activated sludge inoculum with the other constituents of the final mixture. At the end of incubation, after the specified exposure times of usually 3 hours, samples are withdrawn to measure the rate of decrease of the concentration of dissolved oxygen in the cell designed for the purpose (Fig.2 of Annex 3) or in a completely filled BOD bottle. The manner in which the incubations begin also depends on the capacity of the equipment used to measure oxygen consumption rates. For example, if it comprises a single oxygen probe, the measurements are made individually. In this case, the various mixtures needed for the test in synthetic sewage should be prepared but the inoculum should be withheld, and the requisite portions of sludge should be added to each vessel of the series. Each incubation should be started in turn, at conveniently timed intervals of e.g. 10 to 15 minutes. Alternatively, the measuring system may comprise multiple probes that facilitate multiple simultaneous measurements; in this case, inoculum may be added at the same time to appropriate groups of vessels.

37. The activated sludge concentration in all test, reference and blank (but not abiotic control) mixtures is nominally 1.5 g/L of suspended solids. The oxygen consumption should be measured after 3 hours of exposure. Additional 30-minute exposure measurements should be performed as appropriate and previously described in paragraph 5.

Nitrification potential of sludge

38. In order to decide whether sludge nitrifies and, if so, at what rate, mixtures (F_B) as in the blank control and additional 'control' mixtures (F_N) but which also contain N-allylthiourea at 11.6 mg/L should be prepared. The mixtures should be aerated and incubated at 20° ± 2°C for 3 hours. Then the rates of oxygen uptake should be measured and the rate of oxygen uptake due to nitrification calculated.

Test designs

Range-finding test

39. A preliminary test is used, when necessary, to estimate the range of concentrations of the test substance needed in a definitive test for determining the inhibition of oxygen consumption. Alternatively, the absence of inhibition of oxygen consumption by the test substance in a preliminary test may demonstrate that a definitive test is unnecessary, but triplicates at the highest tested concentration of the preliminary test (typically 1000 mg/L, but dependent on the data requirement) should be included.

Table 1 Examples of mixtures for the preliminary test

Reagent	**Original Concentration**				
Test substance stock solution	10 g/L				
Synthetic medium stock solution	See paragraph 16				
Activated sludge stock suspension	3 g/L of suspended solids				
Components of mixtures	Dosing into test vessels (a)				
	F_{T1}	F_{T2}	F_{T3-5}	F_{B1-2}	F_A
Test substance stock solution (mL) (paragraphs 19 to 21)	0.5	5	50	0	50
Synthetic sewage feed stock solution (mL) (paragraph 16)	16	16	16	16	16
Activated sludge suspension (mL) (paragraphs 26 to 29)	250	250	250	250	0
Water (paragraph 15)	233.5	229	184	234	434
Total volume of mixtures (mL)	500	500	500	500	500
Concentrations in the mixture					
Test suspension (mg/L)	10	100	1000	0	1000
Activated sludge (suspended solids) (mg/L)	1500	1500	1500	1500	0
(a) The same procedure should be followed with the reference substance, to give flasks F_{R1-3}					

40. The test should be performed using at least three concentrations of the test substance, for example, 10 mg/L, 100 mg/L and 1000 mg/L with a blank control and, if necessary, at least three abiotic controls with the highest concentrations of the test substance (see as example Table 1). Ideally the lowest concentration should have no effect on oxygen consumption. The rates of oxygen uptake and the rate of nitrification, if relevant, should be calculated; then the percentage inhibition should be calculated. Depending on the purpose of the test, it is also possible to simply determine the toxicity of a limit concentration, e.g. 1000 mg/L. If no statistically significant toxic effect occurs at this concentration, further testing at higher or lower concentrations is not necessary. It should be noted that poorly water soluble

substances, mixtures with components of different water solubility and adsorptive substances should be directly weighed into the test vessels. In this case, the volume reserved for the test substance stock solution should be replaced with dilution water.

Definitive test

Inhibition of total oxygen uptake

41. The test should be carried out using a range of concentrations deduced from the preliminary test. In order to obtain both a NOEC and an EC_x (e.g. EC_{50}), six controls and five treatment concentrations in a geometric series with five replicates are in most cases recommended. The abiotic control does not need to be repeated if there was no oxygen uptake in the preliminary test, but if significant uptake occurs abiotic controls should be included for each concentration of test substance. The sensitivity of the sludge should be checked using the reference substance 3.5-dichlorophenol. The sludge sensitivity should be checked for each test series, since the sensitivity is known to fluctuate. In all cases, samples are withdrawn from the test vessels after 3 hours, and additionally 30 minutes if necessary, for measurement of the rate of oxygen uptake in the oxygen electrode cell. From the data collected, the specific respiration rates of the control and test mixtures are calculated; the percentage inhibition is then calculated from equation 7, below.

Differentiation between inhibition of heterotrophic respiration and nitrification

42. The use of the specific nitrification inhibitor, ATU, enables the direct assessment of the inhibitory effects of test substances on heterotrophic oxidation, and by subtracting the oxygen uptake rate in the presence of ATU from the total uptake rate (no ATU present), the effects on the rate of nitrification may be calculated. Two sets of reaction mixtures should be prepared according to the test designs for EC_x or NOEC described in paragraph 41, but additionally, ATU should be added to each mixture of one set at a final concentration of 11.6 mg/L, which has been shown to inhibit nitrification completely in sludge with suspended solids concentrations of up to 3000 mg/L (4). The oxygen uptake rates should be measured after the exposure period; these direct values represent heterotrophic respiration only, and the differences between these and the corresponding total respiration rates represent nitrification. The various degrees of inhibition are then calculated.

Measurements

43. After the exposure period(s) a sample from the first aeration vessel should be transferred to the oxygen electrode cell (Fig.1of Annex 2) and the concentration of dissolved oxygen should immediately be measured. If a multiple electrode system is available, then the measurements may be made simultaneously. Stirring (by means of a covered magnet) is essential at the same rate as when the electrode is calibrated to ensure that the probe responds with minimal delay to changing oxygen concentrations, and to allow regular and reproducible oxygen measurements in the measuring vessel. Usually, the self-stirring probe system of some oxygen electrodes is adequate. The cell should be rinsed with water between measurements. Alternatively, the sample can be used to fill a BOD bottle (Fig. 2 of Annex 3) fitted with a magnetic stirrer. An oxygen probe with a sleeve adaptor should then be inserted into the neck of the bottle and the magnetic stirrer should be started. In both cases the concentration of dissolved oxygen should continuously be measured and recorded for a period, usually 5 to 10 minutes or until the oxygen concentration falls below 2 mg/l. The electrode should be removed, the mixture returned to the aeration vessel and aerating and stirring should be continued, if measurement after longer exposure periods is necessary.

Verification of the test substance concentration

44. For some purposes, it may be necessary to measure the concentration of the test substance in the test vessels. It should be noted that if stock solutions of:

- poorly water soluble substances,
- mixtures with components with different water solubility, or
- substances with good water solubility, but where the concentration of the stock solution is near the maximum water solubility,

are used, the dissolved fraction is unknown, and the true concentration of the test material that is transferred into the test vessels is not known. In order to characterize the exposure, an analytical estimation of the test substance concentrations in the test vessels is necessary. To simplify matters, analytical estimation should be performed before the addition of the inoculum. Due to the fact that only dissolved fractions will be transferred into test vessels, measured concentrations may be very low.

45. To avoid time-consuming and expensive analytics, it is recommended to simply weigh the test material directly into the test vessels and to refer to the initial weighed nominal concentration for subsequent calculations. A differentiation between dissolved, undissolved or adsorbed fractions of the test material is not necessary because all these fractions appear under real conditions in a waste water treatment plant likewise, and these fractions may vary depending on the composition of the sewage. The aim of the guideline is to estimate a non inhibitory concentration realistically and it is not suitable to investigate in detail which fractions make a contribution to the inhibition of the activated sludge organisms. Finally, adsorptive substances should be also weighed directly into the test vessels; and the vessels should be silanized in order to minimize losses through adsorption.

DATA AND REPORTING

Calculation of oxygen uptake rates

46. The oxygen uptake rates should be calculated from the mean of the measured values, e.g. from the linear part of the graphs of oxygen concentration versus time, limiting the calculations to oxygen concentrations between 2.0 mg/L and 7.0 mg/L, since higher and lower concentrations may themselves influence rates of consumption. Excursion into concentration bands below or above these values is occasionally unavoidable and necessary, for example, when respiration is heavily suppressed and consequently very slow or if a particular activated sludge respires very quickly. This is acceptable provided the extended sections of the uptake graph are straight and their gradients do not change as they pass through the 2.0 mg/L or 7.0 mg/L O_2 boundaries. Any curved sections of the graph indicate that the measurement system is stabilising or the uptake rate is changing and should not be used for the calculation of respiration rates. the oxygen uptake rate should be expressed in milligrams per litre per hour (mg/Lh) or milligrams per gram dry sludge per hour (mg/gh). The oxygen consumption rate, R, in mg/Lh, may be calculated or interpolated from the linear part of the recorded oxygen decrease graph according to Equation 1:

$$R = (Q_1 - Q_2)/\Delta_t \times 60 \qquad (1)$$

where:

Q_1 is the oxygen concentration at the beginning of the selected section of the linear phase (mg/L);
Q_2 is the oxygen concentration at the end of the selected section of the linear phase (mg/L);
Δ_t is the time interval between these two measurements (min.).

47. The specific respiration rate (R_s) is expressed as the amount of oxygen consumed per g dry weight of sludge per hour (mg/gh) according to Equation 2:

$$R_s = R/SS \qquad (2)$$

where SS is the concentration of suspended solids in the test mixture (g/L).

48. The different indices of R which may be combined are:

S specific rate
T total respiration rate
N rate due to nitrification respiration
H rate due to heterotrophic respiration
A rate due to abiotic processes
B rate based on blank assays (mean)

Calculation of oxygen uptake rate due to nitrification

49. The relationship between total respiration (R_T), nitrification respiration (R_N) and heterotrophic respiration (R_H) is given by Equation 3:

$$R_N = R_T - R_H \quad (3)$$

where:
R_N is the rate of oxygen uptake due to nitrification (mg/Lh);
R_T is the measured rate of oxygen uptake by the blank control (no ATU; F_B) (mg/Lh).
R_H is the measured rate of oxygen uptake of the blank control with added ATU (F_N) (mg/Lh).

50. This relationship is valid for blank values (R_{NB}, R_{TB}, R_{HB}), abiotic controls (R_{NA}, R_{TA}, R_{HA}) and assays with test substances (R_{NS}, R_{TS}, R_{HS}) (mg/gh). Specific respiration rates are calculated from:

$$R_{NS} = R_N/SS \quad (4)$$
$$R_{TS} = R_T/SS \quad (5)$$
$$R_{HS} = R_H/SS \quad (6)$$

51. If R_N is insignificant (e.g. < 5% of R_T in blank controls) in a preliminary test, it may be assumed that the heterotrophic oxygen uptake equals the total uptake and that no nitrification is occurring. An alternative source of activated sludge would be needed if the tests were to consider effects on heterotrophic and nitrifying micro-organisms. A definitive test is performed if there is evidence of suppressed oxygen uptake rates with different test substance concentrations.

Calculation of percentage of inhibition

52. The percentage inhibition, I_T, of total oxygen consumption at each concentration of test substance, is given by Equation 7:

$$I_T = [1 - (R_T - R_{TA})/R_{TB}] \times 100\% \quad (7)$$

53. Similarly, the percentage inhibition of heterotrophic oxygen uptake, I_H, at each concentration of test substance, is given by Equation 8:

$$I_H = [1-(R_H - R_{HA})/R_{HB}] / 100\% \quad (8)$$

54. Finally, the inhibition of oxygen uptake due to nitrification, I_N, at each concentration, is given by Equation 9:

$I_N = [1 – (R_T – R_H)/(R_{TB} – R_{HB})] \times 100\%$ (9)

55. The percentage inhibition of oxygen uptake should be plotted against logarithm of the test substance concentration (inhibition curve, see Fig.3 of Annex 4). Inhibition curves are plotted for each aeration period of 3 h or additionally after 30 min. The concentration of test substance which inhibits the oxygen uptake by 50% (EC_{50}) should be calculated or interpolated from the graph. If suitable data are available, the 95% confidence limits of the $EC_{50,}$ the slope of the curve, and suitable values to mark the beginning of inhibition (for example, EC_{10} or EC_{20}) and the end of the inhibition range (for example, EC_{80} or EC_{90}) may be calculated or interpolated.

56. It should be noted that in view of the variability often observed in the results, it may in many cases be sufficient to express the results additionally in order of magnitude, for example:

EC_{50} <1 mg/L
EC_{50} 1 mg/L to 10 mg/L
EC_{50} 10 mg/L to 100 mg/L
EC_{50} > 100mg/L

Interpretation of results

ECx

57. ECx-values including their associated lower and upper 95% confidence limits for the parameter are calculated using appropriate statistical methods (e.g. probit analysis, logistic or Weibull function, trimmed Spearman-Karber method or simple interpolation (11)). An ECx is obtained by inserting a value corresponding to x% of the control mean into the equation found. To compute the EC50 or any other ECx, the per-treatment means (x) should be subjected to regression analysis.

NOEC estimation

58. If a statistical analysis is intended to determine the NOEC, per-vessel statistics (individual vessels are considered as replicates) are necessary. Appropriate statistical methods should be used according to the OECD Document on Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application (11). In general, adverse effects of the test substance compared to the control are investigated using one-tailed (smaller) hypothesis testing at $p \leq 0.05$.

Test report

59. The test report should include the following information:

Test substance

- common name, chemical name, CAS number, purity;
- physico-chemical properties of the test substance (e.g. log Kow, water solubility, vapour pressure, Henry's constant (H) and possible information on the fate of the test substance e.g. adsorption to activated sludge);

Test system

- source, conditions of operation of the wastewater treatment plant and influent it receives, concentration, pre-treatment and maintenance of the activated sludge;

Test conditions

- test temperature, pH during the test and duration of the exposure phase(s);

Results

- specific oxygen consumption of the controls (mg O_2/(g sludge x h);
- all measured data, inhibition curve(s) and method for calculation of EC_{50};
- EC_{50} and, if possible, 95 per cent confidence limits, possibly EC_{20}, EC_{80}; possibly NOEC and the used statistical methods, if the EC50 cannot be determined;
- results for total, and if appropriate, heterotrophic and nitrification inhibition;
- abiotic oxygen uptake in the physico-chemical control (if used);
- name of the reference substance and results with this substance;
- all observations and deviations from the standard procedure, which could have influenced the result.

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ANNEX 1

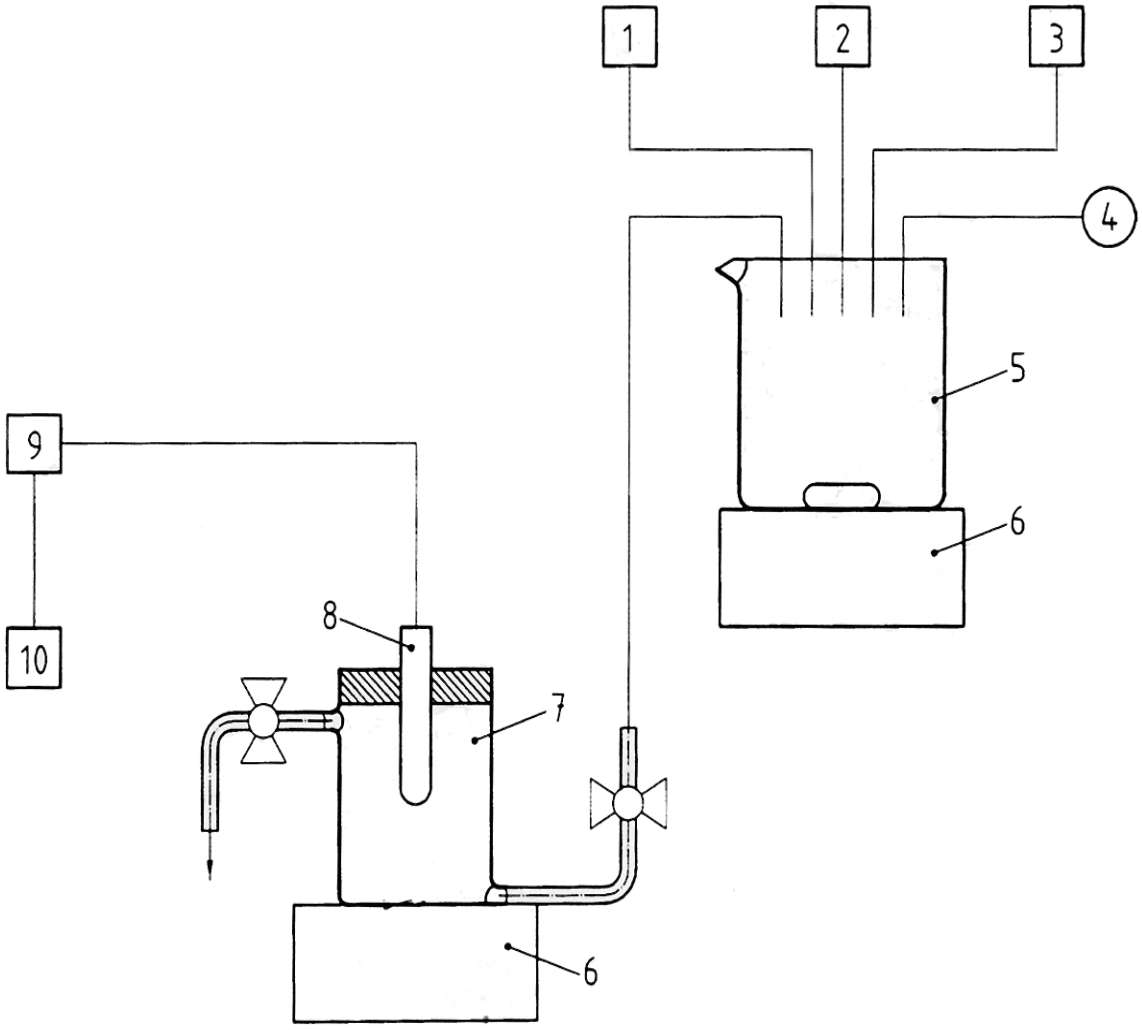
DEFINITIONS

The following definitions are applicable to this Guideline.

NOEC (no observed effect concentration) is the test substance concentration at which no effect is observed. In this test, the concentration corresponding to the NOEC, has no statistically significant effect ($p < 0.05$) within a given exposure period when compared with the control.

ECx (Effect concentration for x% effect) is the concentration that causes an x% of an effect on test organisms within a given exposure period when compared with a control. For example, an EC_{50} is a concentration estimated to cause an effect on a test end point in 50% of an exposed population over a defined exposure period.

ANNEX 2

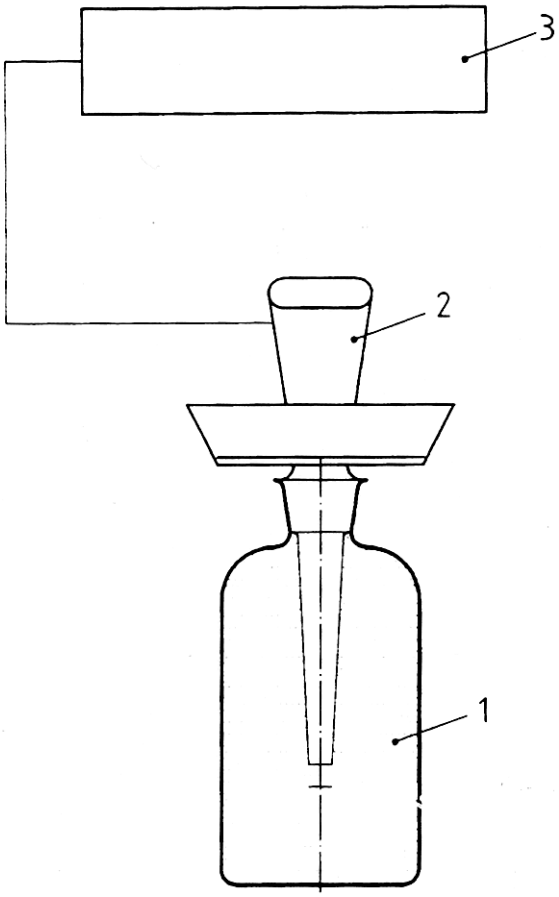


Key

1	activated sludge	6	magnetic stirrer
2	synthetic medium	7	oxygen measuring cell
3	test substance	8	oxygen electrode
4	air	9	oxygen measuring instrument
5	mixing vessel	10	recorder

Fig. 1 Examples for measuring unit

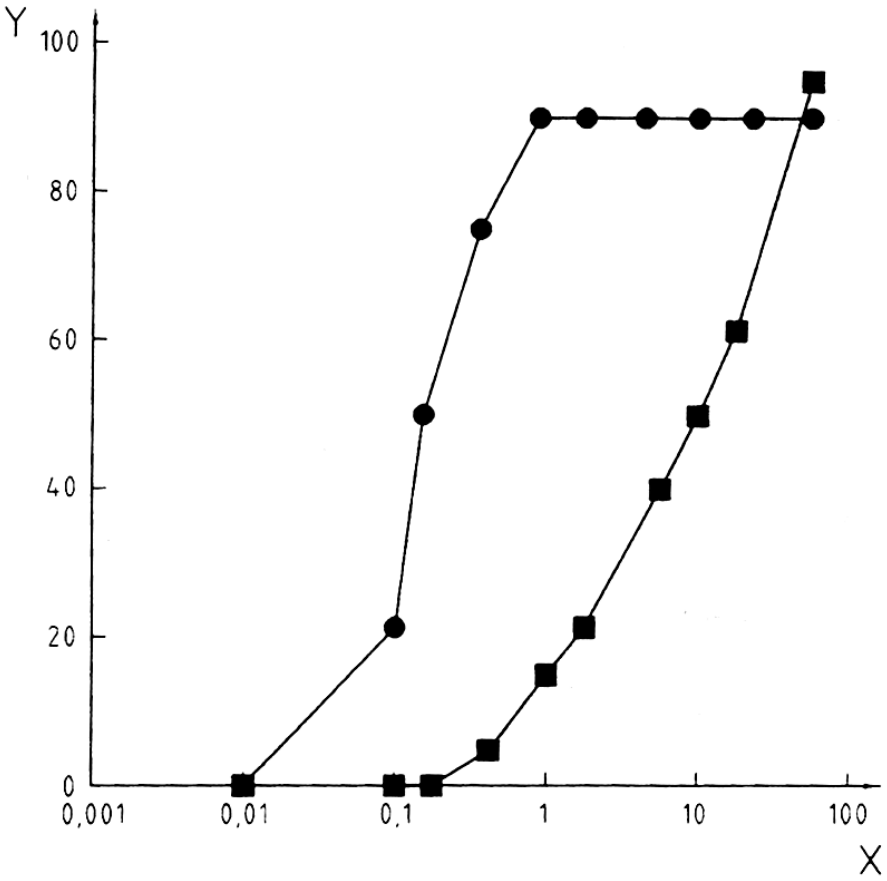
ANNEX 3



Key
1 Test vessel
2 oxygen electrode
3 oxygen measuring instrument

Fig. 2 Example of measuring unit, using a BOD bottle

ANNEX 4



Key

X concentration of 3,5-dichlorophenol (mg/l)

Y inhibition (%)

inhibition heterotrophic respiration

inhibition nitrification

} using a nitrifying sludge

Fig. 3 Example of inhibition curves